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Freeze-thaw survival of sperm from boars without seminal vesicles and bulbourethral glands.

Ronald Malcolm McLaughlin
Iowa State University

FREEZE-THAW SURVIVAL OF SPERM FROM BOARS WITHOUT SEMINAL VESICLES AND BULBOURETHRAL GLANDS

by

Ronald Malcolm McLaughlin

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INTRODUCTION

The purpose of this thesis is to evaluate the freeze-thaw survival of boar spermatozoa that have not contacted secretions of the seminal vesicles and bulbourethral glands during the ejaculatory process. The contributions of these glands to the semen of the boar have been investigated in regard to their influence on the physical and chemical nature of semen (McKenzie, F. F. et al. 1938; Dziuk, P. J. 1958; Mann, T. 1954; Niwa, T. 1961; Niwa, T. et al. 1962; Polge, C. 1956). Other investigators have studied the possibility of alteration of the fertilizing capacity of the spermatozoa that have been separated from seminal plasma (Hess, E. A. et al. 1959; Roy, A. 1955) or that have not contacted various components of seminal plasma (McKenzie, F. F. et al. 1938). It is widely accepted that freeze-thaw survival of porcine spermatozoa is enhanced by separation from seminal plasma through collection of the sperm-rich fraction, through straining or through centrifugation (Roy, A. 1955; Polge, C. 1959; Hoffmann, H. H. 1961; Niwa, T. et al. 1962; Heidrich, H. J. et al. 1964). Some authors have also found that epididymal spermatozoa possess a greater tolerance to cold than ejaculated spermatozoa (Sherman, J. K. 1960; Lasley, J. F. and Bogart, R. A. 1944). This suggested that selective surgical ablation of accessory sex glands might produce a boar that would ejaculate fertile spermatozoa with above normal freezability.

In the transition from epididymal to ejaculated spermatozoa morphologic and metabolic changes occur which are collectively termed maturation. Undoubtedly, many of these changes occur as a continuation of processes initiated in the earlier development of the spermatozoan. However, this thesis proposes that contact with the accessory gland secretions, during ejaculation, is responsible for biochemical changes which may have a profound influence on free-thaw survival of porcine spermatozoa. These changes could come about by alteration of cellular equilibria through addition of substances to the sperm cell or by loss of substances to the seminal plasma or both. Other factors such as pH and osmotic pressure which are altered at various levels of the ejaculatory duct due to the accessory fluids would certainly be capable of influencing this cell membrane transfer and, thus, participate in maturation changes which might alter freeze-thaw survival.

REVIEW OF LITERATURE

Sherman, J. K. (1964) and Smith, A. U. (1961) have presented histories of the development of methods and materials employed in the freezing of semen of the various species. Aamdal, J. (1964), Polge, C. (1959), and Glover, T. D. (1955) have summarized studies on boar semen.

In experimental ablation of the accessory glands of the boar McKenzie, F. F. et al. (1938) performed the surgical procedures on adult boars from which semen had been collected previously to establish their normal semen characteristics. The seminal vesicles were removed through a vertical flank incision and the bulbourethral glands through a horizontal incision ventral to the anus. After a 2 weeks recuperative period collections were resumed to determine what changes had been made in semen characteristics. Their attempts to collect epididymal fluid by cannulating the vasa deferentia were unsuccessful. On postmortem examination they found no evidence of regeneration of glandular tissue.

McKenzie, F. F. et al. (1938) studied the physical nature and sperm content of normal boar semen by changing collection vessels every minute during collection. They found periods of greatest volume output coincident with periods of greatest sperm output. In most boars they noticed two peaks in semen output. The second varied quantitatively among boars, was

absent in some, and tended to disappear with frequent ejaculation. Gelatinous material was ejaculated throughout the period but changed from uniform material of "thick lubricant" consistency, during the early stages, to "typical tapioca material", with or immediately following the high sperm fractions. A clear thin fluid was ejaculated between high sperm fractions. Glover, T. D. (1955) also found the separations between phases to be indistinct due to overlapping. Mann, T. and Glover, T. (1954) found epididymal semen "practically free" from fructose, citric acid, and ergothioneine. Thus, by measuring the concentrations of these compounds in ejaculated semen, they determined that some of the seminal vesicular secretion accompanies the sperm-rich fraction and the remainder is in the post sperm fraction. Niwa, T. (1961) divided ejaculates into five categories showing 1, 2, 3, 4, and irregular peaks of sperm output during a single collection.

In a compilation of 16 papers, Niwa, T. (1961) arrived at the following average values for normal boars:

Volume	200-250 ml
Sperm/ml	$1.0\text{-}2.5 \times 10^{8}$
Sperm/ejaculate	$4.4\text{-}7.0 \times 10^{10}$

For 3 boars without seminal vesicles and bulbourethral glands McKenzie, F. F. et al. (1938) determined the following average values:

Volume	153 cc
Sperm/ml	1.12×10^8
Sperm/ejaculate	1.7×10^{10}

They attributed the low sperm count to inclusion of data from one boar with exceptionally low values.

Routine collection of boar semen is satisfactorily accomplished by training boars to mount a dummy sow (Dziuk, P. J. 1959; Hess, E. A. et al. 1960a; Niwa, T. 1961; Herrick, J. B. and Self, H. L. 1962; Aamdal, J. 1964; and Campbell, E. A. and Lingam, S. A. 1965). According to Niwa, T. (1961), three important considerations in the fabrication of a collecting dummy are variable height, absence of obstruction underneath, and prevention of tipping. Campbell, E. A. and Lingam, S. A. (1965) express a preference for the Japanese style with lateral foot rests over the Norwegian style. The two are otherwise essentially the same.

Polge, C. (1956), Dziuk, P. J. (1958), Niwa, T. (1961), and Campbell, E. A. and Lingam, S. A. (1965) have discussed various aspects of training boars to mount dummy sows. Dziuk, P. J. (1958) substituted a dummy for a live sow after the boars had "established a favorable association with the collection procedure and surroundings". Niwa, T. (1961) and Campbell, E. A. and Lingam, S. A. (1965) smeared dummy sows with urine, vaginal secreta, or semen to induce boars to mount and, thus, circumvented the handling and restraining of a sow.

Hess, E. A. et al. (1960a) considered some boars untrainable. Herrick, J. B. and Self, H. L. (1962) recommend that the same location be used for each collection especially in collecting from shy boars.

Campbell, E. A. and Lingam, S. A. (1965) found collection of a complete ejaculate the first time that a boar mounted a dummy to be of particular significance in the success of the training process.

Glover, T. D. (1955), Polge, C. (1956), Niwa, T. (1961), Aamdal, J. (1964), and Campbell, E. A. and Lingam, S. A. (1965) have discussed the variety of artificial vaginas and their advantages and disadvantages as well as procedural considerations leading to their effective use. Hancock, J. L. and Hovell, G. J. R. (1959) and Herrick, J. B. and Self, H. L. (1962) describe the "gloved hand technique" in which the extended glans penis is grasped with the gloved hand and pressure applied to simulate the cervical constriction of a sow. Advantages of this technique are said to be reduction of contamination and facilitation of fractionation of boar semen during collection.

Dziuk, P. J. (1959) found no difference in fertility between 24 hour and 168 hour collection intervals when semen was stored 48 hours.

Over extended periods of time, Aamdal, J. (1964) and Campbell, E. A. and Lingam, S. A. (1965) have found 72 hours

to be an optimal interval for collection of boar semen. However, Niwa, T. (1961) proposed 5 to 6 days as the shortest interval providing maximum semen quality.

Polge, C. (1956) and Niwa, T. et al. (1962) recovered greater motility from freezing the sperm-rich fraction of the ejaculate than from freezing whole semen. Roy, A. (1955) found that centrifugation of boar semen with removal of seminal plasma increased survival in 4° C. storage. He subsequently employed this preparatory technique in freezing semen and recovered about 50 percent of the originally active spermatozoa. Since this report Polge, C. (1959), Hoffmann, H. H. (1961), Iida, I. and Adachi, T. (1966), and Iida, I. and Ikeda, K. (1966) have removed seminal plasma from boar semen by this technique before freezing, but none of these reports provide information on resulting effects on freeze-thaw survival per se. Self, H. L. (1959) found a significant decrease in post storage motility of centrifuged boar semen over uncentrifuged samples after 3 days and a highly significant decrease after 5 days. Storage temperature was not reported in this article.

Dziuk, P. J. (1958), Hess, E. A. et al. (1959), Hess, E. A. et al. (1960b), and Niwa, T. et al. (1962) have used straining to rid semen of gelatinous material and, thus, facilitate handling. Dziuk, P. J. (1958) found that straining, as compared to decanting, resulted in reduced

motility and recommended decantation immediately after collection as a more efficient procedure.

Estimation of percent motile sperm and a subjective evaluation of type of spermatozoan motility are parameters most commonly considered in determining degree of success in freezing boar semen. Luyet, B. J. and Keane, J. A. (1955) and Iida, I. and Adachi, T. (1966) respectively have used a hemacytometer and a hanging drop method for determining motility. Neither of these methods have claimed popular support. The most often used method for evaluating motility seems to be estimation as described by Herrick, J. B. and Self, H. L. (1962).

Stratman, F. W. et al. (1958) found that percent motile sperm and type of motility were not significantly associated with the percent of fertilized ova in artificial insemination trials with stored (9° C.) boar semen. When fresh semen was used, the association between type of motility and percent of fertilized ova was significant, but association between percent motility and percent of fertilized ova was not.

Stevermer, E. J. et al. (1964) also found motility to be an unrelaible indicator of fertility in artificial insemination of swine.

Herrick, J. B. and Self, H. L. (1962) describe the preparation and application of eosin-nigrosin and "Vital" stain used to differentiate live and dead spermatozoa. Mayer,

D. T. et al. (1951) found that live boar sperm from ejaculated semen took on eosin stain and, thus, could not be distinguished from dead sperm by this method. They also found epididymal spermatozoa to have different staining characteristics which make eosin stains more valid with respect to live vs. dead differentiation. Radford, P. (1961) found it impossible to differentiate live from dead boar sperm using nigrosin eosin stain because dead cells were only faintly stained. Aamdal, J. (1964) reported that he found no significant correlation between percentage of unstained sperm and conception rate using 10 percent anilin-blue which he considers "usable" for differential staining of boar spermatozoa.

Mizuho, A. et al. (1963a) found that diluents with milk and egg yolk sustained motility of boar sperm at above freezing temperatures. They attributed this effect to acceleration of respiration and glycolysis. Milk diluents produced a greater glycolytic acceleration, but egg yolk provided more protection from temperature shock. However, semen was not frozen in this work. Hoffmann, H. H. (1961) tested pasteurized milk, skim milk, and sow milk with and without egg yolk and found them to be inferior to glucose-egg yolk diluent in freeze-thaw survival of boar sperm. Settergren, I. (1959) also reported greater recovery of motile sperm from egg yolk based diluents than from milk based

diluents. Iida, I. and Adachi, T. (1966) used diluents containing 20 percent egg yolk from which the lipid had been partially extracted by ether. In 3 out of 5 diluents that this yolk was used with, motility scores in diluents with yolk were higher after 4 hours at 10° C. plus 5 hours of glycerol equilibration at 5° C. than were motility scores in diluents without yolk, after only 4 hours at 5° C. Although they did not report on controlled experimentation between whole egg yolk and that treated with ether, they suggest that, "It might be advantageous not to eliminate the lipid from egg yolk." No freezing trials were reported on with respect to differential recoveries with or without ether treated egg yolk. Young, D. C. et al. (1957) evaluated 200 semen extenders with respect to their ability to maintain motility of boar spermatozoa. They found those containing 20 percent egg yolk superior to those containing 50 percent.

Dukelow, W. R. and Graham, E. F. (1962) found heated, homogenized whole milk to be superior to an egg yolk-phosphate extender in freezing boar semen. In experiments using tris-buffer as a diluent for freezing boar semen, Benson, R. W. et al. (1967) found that 5 percent egg yolk added no protection against cold shock over that inherent in the tris-buffer.

Hess, E. A. et al. (1959) employed an egg yolk diluent

in freezing boar semen with which they successfully inseminated 7 out of 25 gilts. Hoffmann, H. H. (1961) successfully inseminated 1 out of 11 sows with semen frozen with a egg yolk-glucose diluent. According to Aamdal, J. (1964), Baier, W. (1962) repeated Hoffmann's experiment and succeeded to the extent of 3 conceptions from 43 inseminations. To the author's knowledge these are the only conceptions that have resulted from insemination using frozen boar semen.

Glucose is accepted as being a suitable component in diluents for freezing boar semen (Dziuk, P. J. 1958; Hess, E. A. et al. 1959; Hoffmann, H. H. 1961; Polge, C. 1956). Foley, C. W. et al. (1967) found that glucose diluents maintained a higher level of motility than fructose diluents. Iida, I. and Adachi, T. (1966) and Iida, I. and Ikeda, K. (1966) used sucrose as the carbohydrate component of their diluent but did not give reasons for this choice. Roy, A. (1955) evaluated fructose as a diluent component for freezing boar semen and found that it did not benefit sperm survival. Emmens, C. W. and Blackshaw, A. W. (1950) found pentoses superior to hexoses in maintaining sperm survival in ram, bull, and rabbit semen. The author has not seen reports of this nature with regard to boar semen. As previously noted, the diluents used in successful inseminations with frozen boar semen have been glucose based diluents (Hess, E. A. et al.

1959; Hoffmann, H. H. 1961; Aamdal, J. 1964).

Using 4.5 percent glycine-yolk diluent, Roy, A. (1955) recovered 50 percent motile spermatozoa from boar semen stored at -79° C. for 24 hours. In his hands 4.5 percent glycine was significantly superior to 3 or 6 percent. Settergren, I. (1959) tested glycine-yolk, glucose-yolk, citrate, and milk diluents in freezing boar semen. He found glycine-yolk and glucose-yolk superior to the others and with no significant difference between them. Glycine has also been found to be a beneficial diluent component for storage at above freezing temperatures by Roy, A. (1955), Polge, C. (1956), and Self, H. L. (1959).

Penicillin and dihydrostreptomycin have been shown to enhance survival of boar sperm under various types and durations of storage (Coronel, A. B. and Masankay-Arenas, L. 1954; Dziuk, P. J. and Henshaw, G. 1958; Mizuho, A. et al. 1963a; Lingam, S. A. and Campbell, E. A. 1965). Coronel, A. B. and Masankay-Arenas, L. (1954) considered the beneficial effects due to antimicrobial properties. Whereas, Mizuho, et al. (1963a) attributed the prolonged livability resultant from including these compounds, as well as some sulfas, to an accelerative effect on the metabolism of porcine spermatozoa.

Sherman, J. K. (1964), in discussing factors related to freeze-thaw survival of spermatozoa, considered use of

protective substances and some pertinent aspects of their use. He noted in particular that these substances must not interfere with the functional integrity of cells and that special physiological media may be required by the cells during the pretreatment period prior to cooling.

Polge, C. (1956) used 8 to 10 percent glycerol as a protective agent in freezing boar semen and concluded that the higher level produces sperm damage. In later work (Polge, C. 1957) he found that at 37° C. between 5 and 7.5 percent glycerol is the maximum that boar sperm can tolerate without showing depressed motility. In other freezing trials with boar sperm, Niwa, T. et al. (1962) obtained greatest motility with 7 percent glycerol. Dukelow, W. R. and Graham, E. F. (1962) found 5 to 7 percent superior to 15 to 20 percent. Iida, I. and Adachi, T. (1966) recovered greater percentages of motile sperm after -79° C. storage when 6 and 8 percent glycerol diluents were used than when glycerol levels were at 4 or 12 percent. However, Hess, E. A. et al. (1960b) found no significant difference between a 6 percent glycerol diluent and a 0 percent control with respect to post-thaw sperm motility.

Polge, C. (1959) found boar sperm to be more tolerant of glycerol addition at 15° C. than at higher temperatures. Mizuho, A. et al. (1963c) found the protective substances glycerol, ethylene glycol, and propylene glycol beneficial

at lower temperatures but harmful at higher temperatures.

King, G. J. and Macpherson, J. W. (1966a) evaluated the following methods of adding glycerolated diluents: 1) added in the initial diluent at 33° C., 2) added at room temperature, 3) added as a single portion at 5° C., and 4) added in 4 portions at 5° C. Analysis of their data showed no significant difference among treatments as to effect on subsequent post-thaw motility.

Aamdal, J. (1964) preferred 7 percent glycerol diluents during the freezing of boar semen but found levels above 2 percent to be detrimental for insemination. Hoffmann, H. H. (1961) and Hess, E. A. et al. (1959) employed glycerol as a diluent component in successful inseminations of sows with frozen semen.

Sodium citrate, a compound commonly used in diluents for storing semen of other species, has been shown to be detrimental to motility of porcine semen by Roy, A. (1955), Polge, C. (1956), Settergren, I. (1959), and Dziuk, P. J. (1958). Hess, E. A. et al. (1960a) found that inclusion of sodium bicarbonate in diluents had no significant beneficial effects in respect to duration of motility of boar semen. Polge, C. (1956) cites Milovanov, V. K. (1934) and Selivanova, O. A. (1934) as suggesting that diluting fluids with high proportions of electrolytes are harmful to boar spermatozoa.

In the narrow range between 30×10^6 and 60×10^6 sperm/ml,

Dukelow, W. R. and Graham, E. F. (1962) found that concentration of spermatozoa did not significantly effect freezability. However, Niwa, T. et al. (1962) reported that final sperm concentrations of 2.5×10^8, or higher, yielded maximum freeze-thaw survival. Expression of dilution as the ratio of semen to diluent is commonly used in reporting the effects of dilution. Dziuk, P. J. (1958) recovered greater motility from 1:2, 1:4, and 1:8 dilutions than from 1:1 dilution in storing boar semen at above freezing temperatures.

In freezing boar semen, Polge, C. (1959) found dilutions of 1:2 to 1:5 superior to 1:20 dilution.

Mizuho, A. et al. (1963b) noted that when semen was diluted rapidly, the metabolic activity and motility decreased in proportion to the dilution ratio irrespective of the kind of diluent.

Sherman, J. K. (1964) noted that the rate at which cells are cooled is a vital factor in freeze-thaw survival and, further, that optimal cooling rates differ among cells. Hess, E. A. et al. (1960b) had found loss of motility in boar sperm directly related to rate of temperature decline. Mizuho, A. et al. (1963b) found the significance of rate of cooling to be related to actual temperatures in that boar sperm tolerate greater rates of cooling at high temperatures than at low temperatures.

Niwa, T. (1961) cited earlier work in which he recovered

the greatest number of motile boar sperm after cooling from 25° to 5° C. over a 10 hour period. King, G. J. and Macpherson, J. W. (1966b) cooled boar semen from 33° to 5° C. in 2, 4, and 10 hours and found 4 hours superior to 2 and 10 hours. That the condition of the semen influences the effect of cooling rate was shown by Polge, C. (1956). He found that when undiluted, strained whole semen was cooled to temperatures below 10° C., very few of the sperm could be reactivated. By contrast, if undiluted sperm-rich fraction was cooled to 5° C., the sperm retained their capacity for motility for long periods of time.

The effects of rate of temperature decline on boar semen in the transitional region between above freezing and below freezing have been specifically investigated by Hess, E. A. et al. (1960b) and Iida, I. and Ikeda, K. (1966). In cooling diluted boar sperm from 5° C. to -15° C. in 15, 30, 45, 60, 75, and 90 minutes, Hess, E. A. et al. (1960b) found no significant advantages in times longer than 45 minutes. Yet, 15 and 30 minutes were significantly inferior to all four longer periods. Iida, I. and Ikeda, K. (1966) transferred diluted porcine semen from equilibration at 10° C. to alcohol baths at different low temperatures between -5° C. and -40° C. They found recovery to be best in those transferred to the highest temperature and, also, that recovery in these was not significantly different from recovery in controls frozen from

10° C. to -10° C. at 1° per minute.

With respect to freezing rates, Sherman, J. K. (1964) reported that, "Slower rates have been more beneficial to some cells and tissues, faster rates to others. Each type of material should be evaluated as to its optimal rate rather than accepting that found successful with other material." Work cited in Sherman's article points out the wide variation in susceptibility of spermatozoa of several species to different rates of freezing. In experimentation with boar semen, Settergren, I. (1959) found freezing rates under 3° C./min better than faster rates. Dukelow, W. R. and Graham, E. F. (1962) support this work in finding a 2° C./min rate superior to 5° C./min. Hoffman, H. H. (1961) used a freezing rate of 1° C./min in freezing semen which was later inseminated and resulted in conception.

Differential freezing rates for boar semen are of interest because of interrelationships between actual temperature and rates of cooling and freezing. Niwa, T. et al. (1962) and King, G. J. and Macpherson, J. W. (1966b) are in agreement that greatest recovery is realized in freezing boar semen if rates greater than 1° C./min are used between 5° C. and -10° C. and if rates greater than 1° C./min are used below -30° C. Other temperature ranges and rates of cooling that they used are not alike. Therefore, other conclusions in this respect would be speculative.

The "critical temperature" is defined by Dukelow, W. R. and Graham, E. F. (1962) as the range of temperature over which the freezing rate must be most closely controlled. This range has been described as lying between -15° to -25° C., -10° to -30° C., and -8° to -38° C. by Polge, C. (1957), Niwa, T. et al. (1962), and Dukelow, W. R. and Graham, E. F. (1962) respectively.

Sherman, J. K. (1964) reported that cells vary in the minimum temperature from which they can be revived. An example of interspecies variation is seen in the finding by Polge, C. (1956) that in his experience, boar sperm stored in liquid nitrogen are not easily revived. While on the contrary, bull sperm are easily revived after such storage. The minimum tolerated temperature, according to Sherman, J. K. (1964), is generally the best temperature for storage. The author has not found work oriented toward finding the minimum tolerated temperature for boar semen.

Discussion of cooling and freezing is not complete without mention of methods of decreasing and measuring temperatures. Pickett, B. W. and Komarek, R. J. (1967) froze boar semen in screw cap culture tubes. They measured actual temperature of the semen-diluent mixture with thermocouples. King, G. J. and Macpherson, J. W. (1966a) used a mechanical freezing device, Alcohol and dry ice baths have been used by Iida, I. and Adachi, T. (1966), Iida, I. and Ikeda, K. (1966), and Hess, E. A.

et al. (1960a). Iida, I. and Ikeda, K. (1966) used 1.5 ml ampules with a diameter of 1 cm in which 1 ml of diluted semen was placed. The temperature of the semen was measured by the use of a thermocouple. Iida, I. and Adachi, T. (1966) measured sample temperature and bath temperature and found them to be very close in time. Unfortunately, they did not describe the sample container used. Hess, E. A. et al. (1960b) froze boar semen in 50 ml serum bottles by cooling the alcohol bath with dry ice. They withdrew samples from the bath at 5, -5, -15, -25, -35, and -70° C. (bath temperature) for microscopic evaluation.

Glycerol equilibration periods of 18 and 12 hours were found superior to shorter periods by Polge, C. (1957) and Settergren, I. (1959). Hess, E. A. et al. (1960b) compared motility of boar semen samples stored at -95° C. for 24 hours after being equilibrated for 0, 4, or 24 hours and found no significant differences. Dukelow, W. R. and Graham, E. F. (1962), Iida, I. and Adachi, T. (1966), and King, G. J. and Macpherson, J. W. (1966a) have found equilibration periods in the range of 1 to 6 hours superior to times of 8 to 18 hours.

Roy, A. (1955), Hess, E. A. et al. (1959), Hess, E. A. et al. (1960b), and Niwa, T. (1961) reported using glycerol equilibration temperatures within the 4 to 6° C. range. Iida, I. and Adachi, T. (1966) found 5° C. equilibration

harmful with respect to motility and percentage of motile sperm as compared to 10° and 15° C.

MATERIALS AND METHODS

Preparation of boars

Surgical methods were used in attempts to develop a preparation which would serve as a continuing source of porcine spermatozoa that had not contacted the secretions of the accessory sex glands.

To this end the vasa deferentia were transected outside the external inguinal rings in two adult boars in attempts to collect epididymal spermatozoa by electroejaculation. The ultimate objective was a cannulated boar to serve as a source of epididymal sperm collectable by electroejaculation or through the use of a dummy sow. This effort was discontinued because collection of semen by electroejaculation was not successful in the period of anesthesia in which the surgery was performed. Subsequently, the problem was approached from the point of view of removing rather than bypassing the accessory glands. The prostatic para disseminata and the urethral glands are surgically inaccessible. Their functional removal would necessitate radioisotopic destruction or similar procesures* which were considered beyond the scope of this thesis. Surgical efforts were therefore confined primarily to the ablation of the seminal vesicles and bulbourethral glands.

The seminal vesicles and bulbourethral glands were removed

*M. A. Emmerson, Ames, Iowa. Surgical approaches to the pelvic organs. Private communication. 1967.

from 14 pigs, ranging in weight from 40 to 60 pounds, by two different surgical approaches. Promazine hydrochloride was administered intramuscularly 1 to 2 hours before surgery to facilitate the intravenous injection of the anesthetic agent, thiamylal sodium, which was used at a dosage rate of 5 to 8 mg per pound of body weight to induce anesthesia. Methoxyflurane was administered with an Ohio Chemical Company Model 960 Veterinary Anesthesia Machine to maintain surgical anesthesia. A face mask was devised from a surgeon's latex glove as attempts to pass endotracheal tubes of adequate size proved futile. Incorporation of a stomach tube was deemed necessary to prevent the occurrence of extreme gastric distention which resulted in the deaths of two boars under anesthesia.

A pubic symphyseal approach in which both the seminal vesicles and bulbourethral glands were removed through one incision was utilized in 7 boars. The pelvic urethra was elevated to a position ventral to the split symphysis and the glands removed. After the urethra was replaced, the symphysis was drawn together and fixed with stainless steel suture material. Five of the 7 boars died within 8 days after surgery because of urine retention which resulted from stenosis of the pelvic urethra due to swelling and cicatrix formation. This problem was partially avoided in the remaining 2 boars by the passage of an indwelling urethral catheter which was left in place for 48 hours. When the 2

boars that survived surgery were sacrificed 6 months later, one showed bilateral hydronephritis associated with urethral constriction due to scar tissue in the pelvic canal. Before euthanasia semen was collected from these boars by electro-ejaculation and found to be normal in volume and sperm number for this collection method.

In the remaining 7 boars a paramedian abdominal approach was used to remove the seminal vesicles, and a perineal approach was used to remove the bulbourethral glands. Sodium pentobarbital was used as the anesthetic agent in one of these boars at a dosage rate of 10 mg per pound of body weight. The others were anesthetized with thiamylal sodium and methoxyflurane as described above.

The paramedian abdominal incision through which the seminal vesicles were removed extended anteriorly from the anterior border of the pubis about 5 inches. After the penis and prepuce were reflected laterally, the incision into the peritoneal cavity was made on the linea alba. Posterio-ventral reflection of the bladder and lateral reflection of the ureters exposed the seminal vesicles. The glandular material was dissected away from the duct which was then transected. A second incision was made from the dorsal scrotal border to the ventral extremity of the anal sphincter. Deep tissues were bluntly separated to allow digital entry into the pelvic cavity ventral to the rectum and dorsal to

the pelvic urethra. The fascia between the bulbourethral glands and pelvic urethra was dissected away with an ovariectomy hook. With the glands still attached by ducts and vasculature at their posterior extremity, the anterior ends were reflected posteriorly through the incision. The ducts and associated vasculature were clamped en masse and severed distally with a scalpel. The anal sphincter was ruptured in the course of this procedure in several smaller pigs. Repair was made with heat sterilized silk, and no complications resulted.

Two boars, operated on by this procedure, died before recovery from anesthesia due to acute gastric dilatation of unknown etiology.

The 5 pigs surviving this procedure were communally housed for 7 months before training procedures were initiated.

Training of boars and collection of semen

Four of the surgically prepared boars were trained to mount a dummy sow for semen collection, but electroejaculation was used in the fifth as lameness prevented him from mounting. The training period began approximately 7 months after removal of the seminal vesicles and bulbourethral glands. The boars were moved to communal outdoor quarters adjacent to the building in which collections were to be made. For a period of 1 week each boar was driven to the collection area daily and

allowed to investigate. This familiarized them with the collection area and, also, trained them to leave the pen 1 at a time as selected,with a minimum of herding. At this time no swine were housed in the collection area nor were semen collection artifacts present.

The following week a tranquilized gilt was confined in a metal "shell" when the boars were brought to the collection area (Figure 1). The vicious and noisy reaction of this gilt to restraint, as well as to advances by the boars, discouraged them from mounting. It was decided at this point to proceed with the training program using the dummy in place of the "shell" and the gilt (Figure 2). To this end the gilt received estradiol cyclopentylpropionate intramuscularly, and urine was collected to smear on the dummy as an enticement to the boars. After the first semen collection was made, semen was also smeared on the dummy as an additional enticement for the remaining boars.

Semen was collected from each boar that mounted the dummy on first mounting to establish a favorable association with the area and the dummy. Furthermore, collections were made on the second, fourth, and sixth day after the first collection in an attempt to solidify the pattern of behavior associated with the collection procedure. Warm weather made it necessary several times during the training period to wet the boars to prevent over heating. It was noticed that when

Figure 1. Metal shell designed to contain a gilt in training boars to mount a dummy

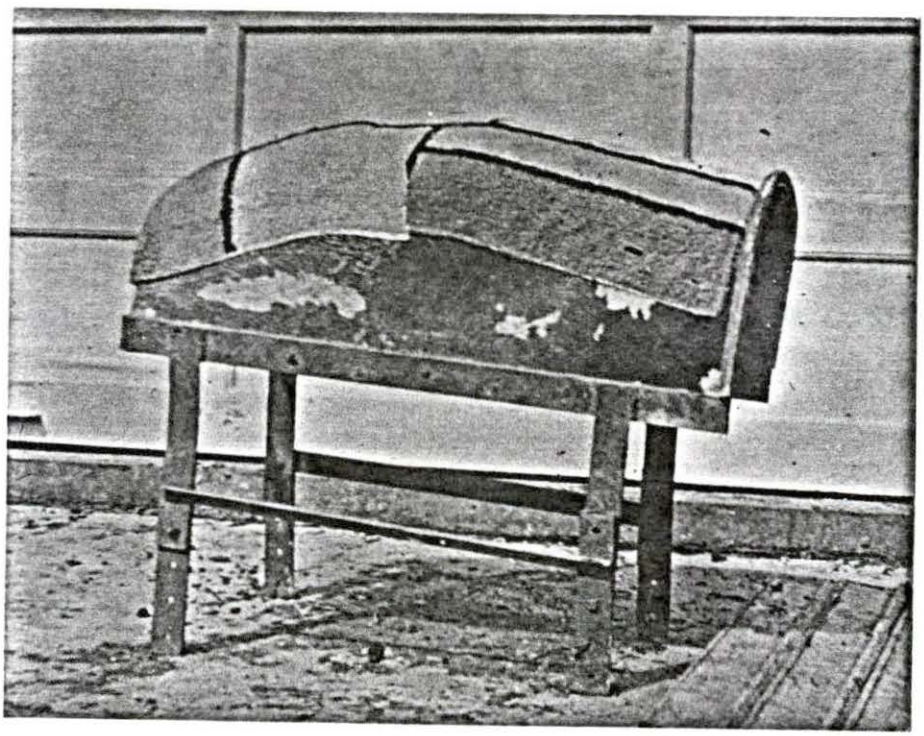
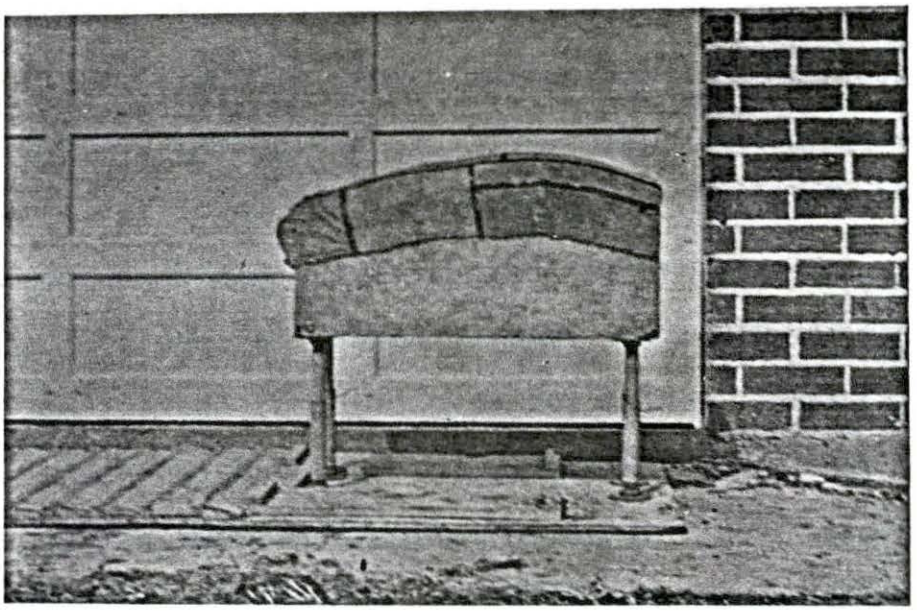


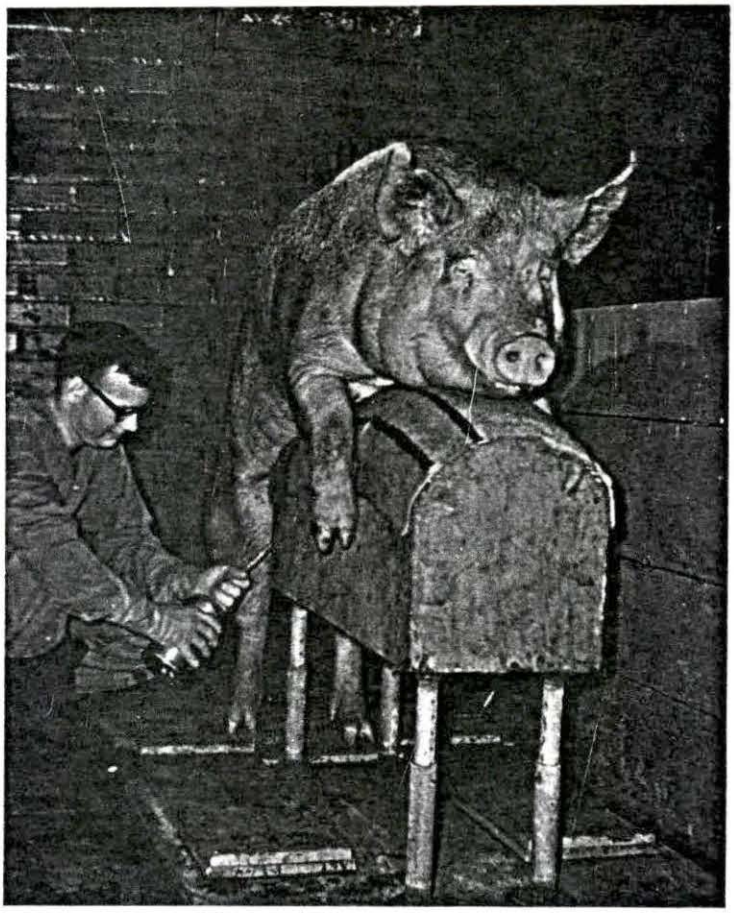
Figure 2. The dummy sow, note full bodied construction and corrugated platform



the boars had been cooled down by this process immediately prior to exposure to the dummy, they mounted more quickly and aggressively. It, also, seemed that their performance was improved if they were confined for a short time near the dummy without access to it.

The observations discussed above and information from work previously cited led to the development of the following regime. The boar to be collected from was driven to the pen next to that containing the dummy. In this pen he was hosed down with cool water for 3 to 4 minutes, or longer if necessary to remove dirt, and dried with towels. If the boars had not urinated and defecated in this time, up to 5 minutes were allowed for them to do so. Access to the dummy was then allowed. All surgically prepared boars that mounted the dummy were collected by the gloved hand technique described earlier. The semen collections were performed on a hollow bodied, adjustable height, Norwegian style dummy mounted on a platform (Figure 3). The entire ejaculate was collected from these boars for evaluation and freezing. As previously mentioned, the remaining surgically prepared boar was collected through the use of an electroejaculator. The normal or control boar, in regular service at the Iowa State University swine nutrition farm, was collected as he mounted sows in estrus. In collecting this boar, efforts were made to exclude the gelatinous material and save the

Figure 3. Semen collection from a surgically prepared boar



sperm-rich fraction of the ejaculate and the clear fluids. In all collections a prewarmed glass vacuum bottle was used to collect and transport semen to the laboratory. In boars 2, 3, and 4 a minimum of 2 days was allowed between collections. The three collections from boars 1 and 5 were made within 5 days.

Evaluation of semen

The percentage of sperm showing active and progressive motility and the percent of sperm alive were estimated as an evaluation of initial semen quality and as a means to quantitate the effect of certain steps in the freezing process. In this thesis the term motility is reserved for this particular type of motility. One drop of semen or diluted semen was placed on a warm microscope slide and a cover slip applied. The slide was then placed on a microscope stage equipped with a stage heater. The sperm in each high power field showing active and progressive movement were counted. Using an estimate of total number of sperm per field in undiluted semen and the dilution factors, approximations of sperm numbers per high power microscopic field were made which allowed computation of the desired percentage. The estimate of the total in undiluted semen was necessary because after the egg yolk diluent was added, only motile sperm could be counted as visibility was obstructed.

The differential stains used to estimate the percent of live sperm were eosin-nigrosin and "Vital" stain. The stains were prepared and used as described by Herrick, J. B. and Self, H. L. (1962).

Sperm concentration was determined through the use of a hemocytometer as described by Herrick, J. B. and Self, H. L. (1962).

Freezing of semen

A diluent and a freezing process were devised to freeze semen collected from the surgically prepared boars and from a normal boar. Two stock solutions were prepared in advance of freezing trials to provide a ready source of diluent. The ingredients of the stock solutions were as follows: Stock solution I - 20 gm glycine, 30 gm glucose, 500 mg dihydrostreptomycin, 10,000 U penicillin, and distilled water to make 1 liter; Stock solution II - 20 gm glycine, 30 gm glucose, 500 mg dihydrostreptomycin, 10,000 U penicillin, 140 ml glycerol, and distilled water to make 1 liter. Six ml of egg yolk were added to 14 ml aliquots of the stock solutions to form diluents I and II respectively. Thus, the diluents contained 1.4 percent glycine, 2.1 percent glucose, and 30 percent egg yolk with diluent II containing, in addition, 14 percent glycerol.

The egg yolk was added 1/2 to 1 hour before the initial

dilution was to be made. Diluent I was warmed to 35° C., and diluent II was cooled to 5° C. so that their temperature would be near that of the semen or diluted semen to which they would later be added.

In some early freezing trials a procedure was used in which nonequilibrated semen was not frozen nor was semen quick frozen in liquid nitrogen. However, in other respects this procedure was the same as the final freezing procedure which is described in this thesis.

Thirty minutes after semen collection was completed, 10 ml of raw semen were added to 5 ml of diluent I which had been warmed to 35° C. Three 5 ml portions of diluent I were added dropwise at 10 minute intervals giving a 1:2 dilution over a 1/2 hour period. The flask containing this mixture was then removed from the 35° C. water bath and placed on an automatic stirrer in a 5° C. refrigerator. The rate of temperature decline was such that the semen-diluent mixture cooled to 14 to 17° C. in 30 minutes. At this time, 20 ml of diluent II were added dropwise in 2 ml portions at 10 minute intervals. After the entire quantity of diluent II had been added, the mixture, then 1:4 dilution, was left on the automatic stirrer for 10 minutes to ensure complete mixing. Samples were then drawn from the flask and pipetted into 2 ml glass ampules, which were heat sealed, to be frozen as nonequilibrated semen. The remaining diluted semen was

equilibrated in the 5° C. refrigerator with constant stirring for 4 hours after which time samples were pipetted to 2 ml glass ampules for freezing as equilibrated semen.

After the ampules were sealed, they were placed in the 5° C. refrigerator for three to five minutes to cool to prevent breakage in freezing. Three methods of freezing were employed in both equilibrated and nonequilibrated semen. One was submerged in liquid nitrogen for 30 minutes. The other two were placed in an acetone bath at 0° C. The bath temperature was reduced at a rate of 1° C. per minute to -40° C. by adding dry ice. When the bath temperature reached -40° C., one sample was transferred to liquid nitrogen for 30 minutes. The other was held at -40° C. in the acetone bath for 10 minutes.

Frozen samples were thawed rapidly by placing ampules in a 35° C. water bath. As soon as the diluted semen had liquified, the ampules were opened and the contents pipetted to warm slides for motility examination or differential staining. Unfrozen samples were pipetted directly to warm slides without prewarming in the water bath.

In the freezing procedure described above, motility and percent live sperm evaluations were made at the following points:

1. Initial evaluation of raw semen.
2. After the addition of diluent I at 35° C.
3. After the addition of diluent II at 10 to 12° C.
4. After quick freezing unequilibrated semen in liquid nitrogen.
5. After freezing unequilibrated semen at -1° C. per minute to -40° C.
6. After freezing unequilibrated semen to -40° C. and super cooling 30 minutes in liquid nitrogen.
7. After 4 hours equilibration in 5° C. refrigerator.
8. After quick freezing equilibrated semen in liquid nitrogen.
9. After freezing equilibrated semen at -1° C. per minute to -40° C.
10. After freezing equilibrated semen to -40° C. and super cooling 30 minutes in liquid nitrogen.

In the case of the procedure in which nonequilibrated semen was not frozen, evaluations 3, 4, 5, and 6 were not performed.

Statistical analysis

Comparisons of sample means for unpaired observations with equal variance and unequal sample size were made between motility means of control and test boar sperm. The same techniques were applied to comparisons of mean motility differences between processing stages, between groups, and within groups (Steel, R. G. D. and Torrie, J. H. 1960). Because of small sample numbers, significance at the 0.1 probability level is reported.

Linear regression was used to determine correlation coefficients between percent of unstained sperm and percent or sperm showing active and progressive motility.

RESULTS

The raw motility percentages and percent of live spermatozoa from control and test boars at various stages of the diluting and freezing process are shown in Table 1. The means of these motilities are in Table 2 and Figure 4. Comparisons between motility means within the two groups which showed statistically significant differences are as follows:

Initial test semen	vs.	first dilution test semen	$P<0.1$
First dilution test semen	vs.	second dilution test semen	$P<0.01$
Second dilution test semen	vs.	equilibrated test semen	$P<0.05$

The comparisons of motility means showing significant differences between the control sperm and test sperm are as follows:

Equilibrated control semen frozen 1°/min to -40° C.	vs.	Equilibrated test semen frozen 1°/min to -40° C.	$P<0.01$
Nonequilibrated control semen frozen 1°/min to -40° C.	vs.	Nonequilibrated test semen frozen 1°/min to -40° C.	$P<0.01$

The coefficients of correlation of sperm motility on percent of sperm unstained by eosin-nigrosin and "Vital" stain were found to be 0.670 and 0.832 respectively.

Table 1. 10 point standard motility and % live sperm during processing

	Motility	Stain	Control			Boar Number 1			Boar Number 2		
1	Initial	motility	85	80	65	70	85	80	90	60	40
		% live Eosin	76	23	46	20	59	25	21	28	65
		% live Vital	82	93	92	85	90	65	86	86	90
2	Dilution I	motility	82	75	70	65	60	50	80	60	60
		% live Eosin	69	18	42	30	48	56	60	42	47
		% live Vital	83	82	91	82	82	42	93	77	79
3	Dilution II	motility	80	70	60	45	50	0	50	60	
		% live Eosin	79	55	77	2	57	15	40	0	
	Unequilibrated	% live Vital	80	65	82	5	76	0	74	42	
4	Quick freeze	motility	0	0.5	0.5	0	0	0	0	0	
		% live Eosin	0	0	0	1	1	0	0	0	
		% live Vital	11	3	0	0	0	0	1	2	
5	1°/min to -40°	motility	2	1	5	0	0	0	1	1	
		% live Eosin	28	0	4	0	0	0	1	0	
		% live Vital	40	2	1	0	2	0	5	0	
6	1°/min to -40°, liquid N_2	motility	1	0.5	3	0	0	0	0.5	0.5	
		% live Eosin	7	1	1	0	0	0	2	0	
	Equilibrated	% live Vital	9	0	0	2	3	0	3	1	
7	Before freeze	motility	90	55	55	1	0.5	0	50	55	8
		% live Eosin	90	52	67	54	42	19	21	41	58
		% live Vital	76	60	95	89	73	57	23	80	70
8	Quick freeze	motility		0.5	0	0	0	0	0	0	
		% live Eosin		1	0	1	0	0	0	0	
		% live Vital		0	0	0	2	6	0	0	
9	1°/min to -40°	motility	6	7	2	0	0	0	0.5	4	0
		% live Eosin	18	2	2	0	0	0	1	0	1
		% live Vital	31	1	2	0	1	1	2	0	4
10	1°/min to -40°, liquid N_2	motility	4	1	2	0	0	0	0.5	2	
		% live Eosin	15	1	1	0	1	1	2	1	
		% live Vital	22	1	1	6	3	3	0	3	

Table 1 (Continued)

	Motility		Stain	Boar Number 3			Boar Number 4				Boar Number 5		
1	Initial	motility		70	90	80	90	80	88	75	70	80	70
		% live	Eosin	10	40	32	22	19	50	2	25	46	29
			Vital	52	91	86	95	87	94	76	75	88	75
2	Dilution I	motility		55	90	65	45	65	80	40	65	75	60
		% live	Eosin	60	37	66	62	61	62	65	32	59	43
			Vital	74	93	83	88	80	85	79	81	95	75
3	Dilution II	motility		40			11				50	55	60
		% live	Eosin	70			65				34	47	12
	Unequilibrated		Vital	81			79				62	73	0
4	Quick freeze	motility		0.5			0				0	0	0
		% live	Eosin	1			0				0	0	0
			Vital	2			0				0	0	0
5	1°/min to -40°	motility		0.5			0				0.5	0	0.5
		% live	Eosin	3			0				0	1	0
			Vital	4			2				0	0	0
6	1°/min to -40°, liquid N_2	motility		0.5			0				0.5	1	0.5
		% live	Eosin	2			0				1	0	0
	Equilibrated		Vital	0			0				1	0	0
7	Before freeze	motility		33	5	60	0	21	18	14	40	45	30
		% live	Eosin	65	80	0	48	57	63	61	45	57	35
			Vital	92	72	60	63	78	52	80	78	77	60
8	Quick freeze	motility		0.5			0				0	0	0
		% live	Eosin	0			0				0	1	0
			Vital	0			0				0	0	0
9	1°/min to -40°	motility		2.5	0	0.5	0	2	0.5	0	0	1	1
		% live	Eosin	5	24	5	3	40	1	6	0	0	0
			Vital	6	87	7	4	55	2	22	1	7	5
10	1°/min to -40°, liquid N_2	motility		7.5	0	0.5	0	0	0	0.5	0	0.5	2
		% live	Eosin	2	3	1	1	2	3	4	0	0	1
			Vital	5	9	8	2	3	0	4	2	0	6

Table 2. Means of observations on active and progressive motility of a normal boar and five boars without seminal vesicles and bulbourethral glands

	Normal Boar		Test Boar	
Initial	76.7		73.7	
Diluent I added	75.7		63.4	
Diluent II added	70.0		42.1	
Equilibration	66.7		23.7	
	Equil	Nonequil	Equil	Nonequil
Quick freeze in liquid N_2	0.2	0.3	0.1	0
1° C/min to -40° C.	5.0	2.7	0.8	0.4
30 minutes in liquid nitrogen from -40° C.	2.3	1.5	0.8	0.4

Figure 4. Semilogarithmic graph, Mean motility of test and control semen vs. time

A. Raw semen

B. After first dilution

C. After second dilution

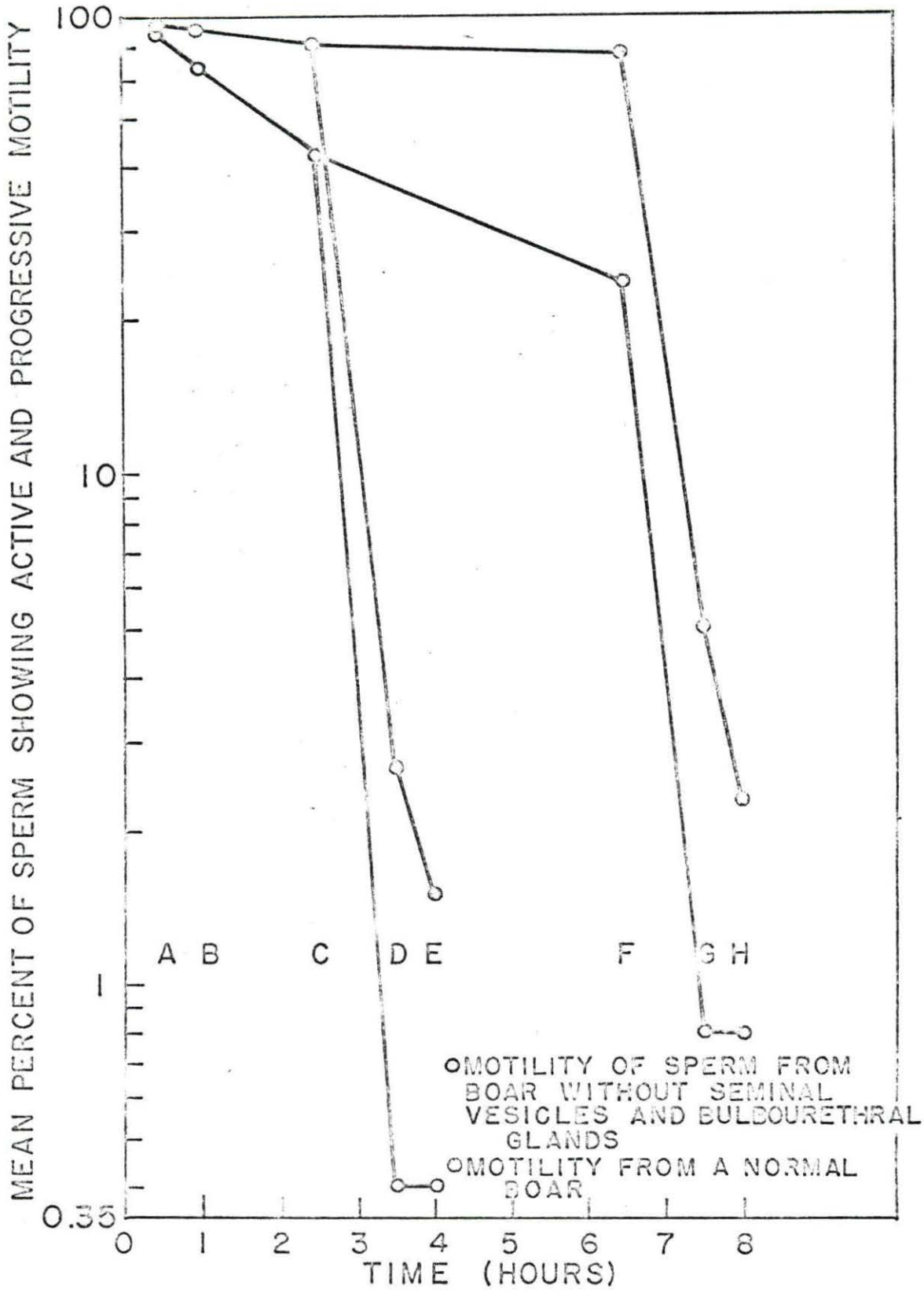
D. Frozen 1° C. per minute to -40° C. and held there 10 minutes

E. Frozen 1° C. per minute to -40° C. then placed in liquid nitrogen for 30 minutes

F. After glycerol equilibration

G. Frozen 1° C. per minute to -40° C. after equilibration and held there 10 minutes

H. Frozen 1° C. per minute to -40° C. after equilibration then placed in liquid nitrogen for 30 minutes


MEAN PERCENT OF SPERM SHOWING ACTIVE AND PROGRESSIVE MOTILITY
100
10
1
0.35
A B C D E F G H
MOTILITY OF SPERM FROM BOAR WITHOUT SEMINAL VESICLES AND BULBOURETHRAL GLANDS
MOTILITY FROM A NORMAL BOAR
0 1 2 3 4 5 6 7 8
TIME (HOURS)

The means of volume, sperm concentration, and number of sperm per ejaculate from boars without seminal vesicles and bulbourethral glands used in this thesis are compared with like parameters of other boars without these glands and of normal boars in Table 3. This shows that these values for test boars are within ranges for normal boars.

The ejaculate from the test boars began as a clear fluid, abruptly became cloudy, and then gradually turned clear again near the end of the collection period. Fractionation of the ejaculate was not performed. No pathological deviations from normal were observed in the ejaculatory capability of these boars.

Table 3. Semen parameters showing: A. Means of 13 samples from four test boars without seminal vesicles and bulbourethral glands, B. Compilation from Niwa, T. (1961) containing results from 16 reports, number of boars not reported, C. Means of 32 samples from three boars without seminal vesicles and bulbourethral glands from McKenzie, F. F. et al. (1938)

Parameter	A	B	C
Mean volume	221 ml	200-250 ml	153 ml
Mean sperm concentration	2.1×10^8/ml	$1.0\text{-}2.5 \times 10^8$/ml	1.1×10^8
Mean sperm per ejaculate	4.6×10^{10}	$4.4\text{-}7.0 \times 10^{10}$	1.7×10^{10}

DISCUSSION

Test semen showed a strong tendency to decrease in motility faster than control semen in all phases of the freezing process. Likewise, it can be seen that in frozen control semen the nonequilibrated samples tend to have a lower motility than equilibrated samples.

The absence of beneficial effect from glycerol equilibration within control and test semen was contrary to what was anticipated. Other workers have used total motility as the criterion for evaluating the effect of equilibration. This quantity was not measured when data were collected for this thesis. Thus, the possibility cannot be ruled out that glycerol equilibration may be beneficial for total motility and at the same time detrimental to active and progressive motility. Another possibility is that the time, 4 hours, is proportionately more detrimental to active and progressive motility than it is to total motility.

The results of the freezing trials indicate that gel-free whole semen from the control boar has a greater freeze-thaw survival than semen collected from test boars without seminal vesicles and bulbourethral glands. Yet, it was expected that test semen would behave like epididymal semen. Niwa, T. et al. (1962) found that epididymal sperm had a higher freeze-thaw survival than the sperm-rich fraction of the

ejaculate, which in turn had a higher freeze-thaw survival than gel-free whole semen. As pointed out in the introduction, others have concurred with these findings.

Thus, it seems that the situation presents itself in which sperm that have not contacted accessory gland secretions, i.e., epididmyal sperm, have a greater freeze-thaw survival than sperm that have been ejaculated in contact with these secretions and are subsequently separated from them by centrifugation. Yet, these ejaculated and centrifuged sperm survive freezing better than ejaculated sperm that have been frozen in contact with accessory fluids, such as sperm from the control boar. All of the above sperm apparently have a higher freeze-thaw survival than the test boar sperm that have not contacted seminal vesicular fluid nor the secretions of the bulbourethral glands.

It appears then, that removal of the seminal vesicles and/or bulbourethral glands has in fact removed some factor or factors from the semen which are beneficial to freezability. Thus, a conditional statement evolves, if seminal plasma is detrimental to freeze-thaw survival, and if secretions of the seminal vesicle and/or bulbourethral glands are beneficial, then some other factor or factors in semen is detrimental to freezability.

Because of the increasing freeze-thaw survival observed with decreasing degree of contact with seminal plasma and the

observation that seminal vesicular fluid and/or bulbourethral gland secretion are not detrimental it seems that the source of detrimental factors must be the urethral and/or prostate glands. The detrimental effect observed may be due to chemical qualities of their secretions or to increased quantities of secretion.

The test boars in this experiment differ from those of McKenzie, F. F. et al. (1938) in that normal sperm numbers and volume were attained and the ejaculatory capability was not damaged. These differences may be partially explained in that those boars used in this thesis were prepared at an immature age and were allowed over seven months recovery. Whereas, McKenzie's were operated as adults and only two weeks were allowed for recovery.

Since the possibility of glandular regeneration in this experiment must be evaluated an addendum on postmortem examination of the test boars will be supplied when all experimentation with them is completed.

Bennett, J. P. and Rowson, L. E. A. (1963) and Amann, R. P. et al. (1963) have successfully cannulated the vas deferens of bulls so that epididymal sperm are collectable by electroejaculation or through the use of an artificial vagina. There does not seem to be an approach suitable for permanent cannulation of the vas deferens in boars. Failure of electroejaculation to extrude epididymal sperm through the transected vas deferens in this experiment is not understood.

Experiences encountered in training the test boars to mount the dummy for semen collection have shown that the presence of a sow can be detrimental. At the same time urine from a sow, not necessarily in estrus, smeared on the dummy appeared to be highly beneficial in the training process. Each of the four boars trained exhibited an individual degree of inclination to mount. These varied from one that mounted without overt preliminary activities to one that would not mount until he had inspected all sides of the dummy and much of the surrounding area. The latter sometimes sat on his haunches with his forefeet on the dummy while his semen was being collected.

It was observed that their interrelationships followed the same behavior pattern, with the more aggressive being the riders, pushers, and fighters. Homosexual behavior was frequently observed. Everytime a boar was returned to the pen after semen was collected, he became the object of sexual attention. Pederastic attempts were not uncommon but were never observed to be completed due to loud and frantic objection of the mounted boar.

Once established the collection routine progressed smoothly. No boar that had mounted the dummy once ever refused to mount at a later date. Collection came to be anticipated as evidenced by fighting among the boars for first position at the gate when the author and assistant

appeared. The boar that was repeatedly collected by electroejaculation did not participate with this enthusiasm. However, he did not become noticably resentful.

Pulsating pressure on the glans penis was not necessary to stimulate ejaculation. However, it was noticed that if pulsating pressure was applied before a boar dismounted after one ejaculatory cycle that another cycle could be obtained.

The full bodied construction of the dummy proved to be obstructive to the collector as well as a potential source of penile injury. When a boar had fully mounted, it was necessary that the penis be directed at a right angle from the body axis to prevent abrasions from the dummy. The most beneficial design feature of this dummy was the platform which was corrugated to provide stable footing for the boars and which, because of the boars' weight, made the dummy practically immovable when in use.

Evaluation is one of the more critical problems encountered in work with boar semen. Though the ultimate criterion is fertility, it was not feasible to make this evaluation on the samples collected. One problem is the lack of availability of females in the appropriate stage of estrus at the time of collection.

Motility evaluation, live-dead differential staining, sperm morphology evaluation, and characterization of physical properties of semen have evolved as fertility test substitutes.

To the author's knowledge active and progressive motility is the only one of these parameters that has been shown to be significantly correlated to fertility of boar semen, and this was found to be true only in fresh semen (Stratman, F. W. et al. 1958). As to live-dead stains, when a student microscope was used the percent live estimates were much higher in relation to motility than when a binocular microscope equipped with an American optical company ortho-illuminator was used. The latter made it easier to discern color variations in stained sperm smears.

Morphological examination disclosed that a disproportionate number of sperm with bent tails, apparently at the site of a cytoplasmic droplet, were left unstained. This became more obvious after the later stages of the freezing process. It was not noticed that these cells displayed greater resistance to freezing than normal cells with respect to maintaining motility capabilities.

Unfortunately, no adequate laboratory test for determining the fertility of frozen boar semen exists.

A great weakness in the literature concerned with the freezing of boar semen is the inadequate reporting of temperatures to which sperm are exposed. Hess, E. A. et al. (1960b) froze boar semen in 50 ml serum bottles placed in an alcohol bath which was cooled at a rate of 1° per minute by the addition of dry ice. Samples were removed at various tempera-

tures between 5 and -70° C. for microscopic examination. Obviously the sperm at the center of this large container would be insulated from the bath temperature and would not reach the temperature described. If the bath temperature were held at a given point long enough, the sperm would eventually reach the minimum temperature but certainly at a much slower rate. Others have not bothered to describe the container used nor the method or rate of temperature reduction. Ideally, a small bore thin-walled container would be best for work intended to evaluate rates of cooling, minimum tolerated temperature, and thawing rates.

It is difficult to correlate the extreme variability in diluent, cooling rates, etc. which have been used in attempts to freeze boar semen.

Conflicting reports lead to difficult decisions as to methods which should be employed in experiments. For example, Hafez, E. S. E. (1962) explains that light is harmful to boar semen because it results in H_2O_2 production. Yet, Marsh, H. M. et al. (1964) found light to have little influence on motility, pH, or oxygen consumption by boar semen. Other conflicting reports are mentioned in the Literature Review.

SUMMARY AND CONCLUSIONS

Surgical removal of seminal vesicles and bulbourethral glands from boars resulted in a loss in freeze-thaw survival of sperm. From the experimental evidence presented, it seems that seminal vesicular and/or bulbourethral fluids benefit freeze-thaw survival, and/or, that prostatic and/or urethral gland fluids are detrimental to freeze-thaw survival.

Semen was evaluated at 10 points in the freezing process by estimation of percent of sperm showing active and progressive motility and by differential staining. Live-dead evaluation using a fast-green stain, "Vital stain," was found more closely correlated to active and progressive motility than was that using eosine-nigrosin stain.

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